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Prospecting *Bacillus* Species Isolated from Rhizosphere of *Calotropis* Plant for Biodegradation of Polycyclic Aromatic Hydrocarbons

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Polycyclic aromatic hydrocarbons are accumulated in the environment due to a variety of anthropogenic activities. In this study, 330 bacterial strains isolated from rhizosphere of *Calotropis sp.*, which were growing in industrial, farming and natural sites, were investigated for their ability to grow in the presence of polycyclic aromatic hydrocarbons added to minimal salt basal medium. Four isolates were found to be capable of using naphthalene, anthracene, chrysene, benzene, toluene and xylene as sole source of carbon. The taxonomic position of these isolates was examined by amplification and sequencing of 16S rDNA gene. The isolates were found to be belonging to *Bacillus* sp. The mean growth rate constant (K) of isolates were found to increase with successive increase in substrate concentration (0.5 to 1.0 mg/50ml). KS37 (*B. cereus PPB6)* degraded 81.39% of anthracene while, KS141 (*Bacillus* sp.*WJ21*) and KS146 (*Bacillus* sp. *MC-BAC2*) degraded 65.30 %, 69.71 % of naphthalene respectively after 6 days of incubation as determined by HPLC analysis. The results obtained indicate that the rhizosphere of *Calotropis* is a rich source of *Bacillus* sp., which have potential to degrade the PAH molecules.

Key words: *Bacillus, Calotropis,* Polycyclic aromatic hydrocarbon, 16S rDNA genes, Rhizosphere.

Environment contaminated with polycyclic aromatic hydrocarbons (PAHs) is considered hazardous as studies using animals have shown the specific carcinogenic, mutagenic and teratogenic effects of some PAHs[1]. Although, some physical processes such as volatilization, leaching and chemical oxidation are often effective in reducing the environmental level of PAHs[2]. Biodegradation using microorganisms is usually the preferred and major route of PAH removal from contaminated environments because of some inherent advantages such as its cost effectiveness and more complete cleanup[3].

Since PAHs are hydrophobic compounds with low solubility in water, they have a propensity to bind with organic matter or soil, limiting their availability to microorganisms. Even with these properties, many bacterial strains have been isolated for their ability to transform, degrade and utilize PAHs as a sole source of carbon and energy[4]. Significant bacterial communities with ability to degrade PAH in soil play a crucial role in biodegradation in spite of their low bioavailability. Microorganisms inoculated into PAHs contaminated soil environments must find and mobilize PAH before degradation and hence motility and hydrophobicity are thought to be

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desired properties[5]. However, the hydrophobicity and motility can potentially increase the ability of bacteria to access PAH within soil[6]. Plant associated bacteria, such as rhizospheric bacteria have been shown to contribute to biodegradation of toxic organic compounds in contaminated soil and could have potential for improving phytoremediation[7]. When a suitable rhizospheric isolated strain is introduced together with a suitable plant, it is inhabited on the root along with indigenous population, thereby enhancing the bioremediation process.

Although, there is enough literature available for the pharmaceutical, ayurvedic and medicinal use, but no information about rhizosphere community of *Calotropis* sp., its molecular characterization and their utilization in sustainable agriculture, biofertilization and ecorestoration has been carried out. Consequently, the current study was designed to study the biodegradation ability of PAH by rhizospheric bacteria isolated from the rhizosphere of *Calotropis* sp. from north and central zone of India.

MATERIALS AND METHODS

Soil samples were collected from the rhizosphere of *Calotropis* plant growing in north and central region of India, from the depths 0-60 cm using an ethanol disinfected scoop.

Total culturable heterotrophs were grown by spray plate technique[8] using minimal salt basal medium (MSB). Liquid hydrocarbon substrates were provided as a vapour phase in a wax sealed desiccators[9]. Cell surface hydrophobicity of degraders was determined by their adherence to hydrocarbons which is based on the partitioning of cells in a two phase system. Growth profile of isolates in anthracene or naphthalene amended medium was determined. Minimum salt basal medium (MSB) was supplemented with different concentrations (0.5, 0.8 and 1.0 mg/50ml) of anthracene or naphthalene. Positive control was experimented in parallel comprising dextrose (2 %) as sole source of carbon. Growth was assessed by measuring OD at 600nm after time interval of 3 h. Mean growth rate (K) was calculated by formula given as:

$$K = 3.322 \log Z_t - Z_0/\Delta T$$

Where K is mean growth rate constant, Z_t is final growth at time t, Z_0 is initial growth at time 0 and ÄT is difference in time.

Residual amount of anthracene and naphthalene was determined by high performance liquid chromatography (HPLC, Shimadzu equipped with UV –Vis detector operating at 254 nm) analysis in culture medium for quantitative estimation of PAH degradation. Genus level identification was carried out by subjecting the bacterial isolate to microscopic, morphological observation, biochemical and physiological characterization. Total genomic DNA for PCR reactions was extracted from an overnight culture using Ultra Pure genomic DNA spin minipreps Kit from Bacterial (Medox) using manufacturer's instructions. PCR amplification of 16SrDNA was carried out in a Thermal Cycler (Eppendorf MC) using eubacterial primers fD1 and rp2 primer set[10]. Taxonomic identification was performed comparing the partial 16S rDNA sequences of isolates[11]. The phylogenetic tree was constructed by the neighbour joining method using the distance matrix from the alignment[12]. The data was statistically analysed for standard deviation and standard error of three independent observations.

RESULTS

It is quite evident from the data presented in table1 that four isolates were found to utilize the entire hydrocarbon *viz.*; chrysene, benzene, toluene and xylene as sole source of carbon in the study (Table 1).

All selected isolates were gram positive, motile, facultative/ aerobic rods which grew at room temperature and alkaline pH. According to the morphological and biochemical properties, isolates showed similarity towards *Bacillus* sp. In addition, phylogenetic characterization indicated that all selected strains were belonging to the *Bacillus* sp.

In the present study, *Bacillus* sp. (KS141) and *B. cereus* (KS37) exhibited positive hydrophobic response (49% and 25%) respectively (Table 1). However, the mean growth rate constant (K) of all the isolates were found to increase with the concentration of substrate. The K value of *B. cereus* (KS1), *B. cereus* (KS37), *Bacillus* sp. (KS141), *Bacillus* sp. (KS146) in medium amended with anthracene (1 mg/50ml) was obtained as 0.45,

Table 1. Growth test on different liquid and solid hydrocarbon and % hydrophobicity

S. No.	Strain	Polyaromatic Hydrocarbon (PAH)						% of Hydro.[g]
		Naph.[a]	Anth.[b]	Chr.[c]	Ben.[d]	Tolu.[e]	Xyl.[f]	
1	KS1	++	++	++	-	+	++	12±1.52
2	KS37	+++	++	+	++	-	-	25±4.40
3	KS141	++	+++	+++	++	+++	++	49±7.80
4	KS146	++	++	+++	+	++	+++	22±1.15

a) naphthalene, b) anthracene, c) chrysene, d) benzene, e) toulene, f) xylene, g) % of hydrophobicity, +++ excellent growth; ++ moderate growth; + weak growth;
– no growth, numerical values are mean ± SD of three independent observations

Table 2. Mean growth rate constant of isolates at varying concentration of substrate

Strain	Mean growth rate constant (K) h^{-1}						
	Anthracene Concentration (mg/ 50ml)			Naphthalene Concentration (mg/50ml)			
	0.5	0.8	1	0.5	0.8	1	Control
KS1	0.25±.026	0.34±.020	0.45±.020	0.26±.020	0.31±.015	0.41±.025	0.69±.017
KS37	0.35±.012	0.39±.017	0.49±.008	0.27±.008	0.29±.014	0.36±.012	0.67±.006
KS141	0.32±.015	0.31±.046	0.35±.020	0.37±.020	0.39±.023	0.45±.011	0.72±.013
KS146	0.31±.008	0.34±.020	0.39±.020	0.26±.020	0.36±.020	0.39±.011	0.66±.030

numerical values are mean ± SE of three independent observations

0.49, 0.35, and 0.39 h^{-1} respectively, which was relatively higher than other concentrations tested and analogous results were obtained for naphthalene (1 mg/50ml). However, the mean growth rate of all the isolates was relatively higher in glucose amended medium, where it was 0.69, 0.67, 0.72, and 0.66 h^{-1} for *B. cereus* (KS1), *B. cereus* (KS37), *Bacillus* sp. (KS141) and *Bacillus* sp. (KS146) respectively as shown in Table 2.

The growth profile of the selected isolates at different concentration of naphthalene and anthracene with respect to control is given in Fig 1.

All the four isolates were found to reduce the anthracene and naphthalene concentration in minimum salt basal medium as evidenced by HPLC analysis. *Bacillus cereus* (KS37), and *Bacillus* sp. (KS146) resulted in 81.39%, and 27.34% degradation of anthracene respectively (Fig 2 a), while 69.71% and 65.30% reduce in naphthalene concentration was observed by *Bacillus* sp. (KS146) and *Bacillus* sp. (KS141) after 6 days respectively (Fig 2 b).

These four isolates (KS1, KS37, KS141 and KS146) were identified as Bacillus sp. using 16SrDNA gene sequence analysis (Fig. 3). The 16S ribosomal RNA gene sequence of isolates KS1, KS37, KS141 and KS146 were submitted to NCBI gene bank and accession no JQ912681, JQ912677, JQ912678, JQ912679 were assigned respectively.

DISCUSSION

The objective of the present investigation was to isolate potential PAHs degrading bacterial isolates from the rhizospheric soil of *Calotropis* sp. A very important finding with respect to the total percentage of degraders was revealed from the present study. It was found that the dominant PAHs degrading bacterial isolates were obtained from industrial sites of all the zones. Earlier also, Hassan et al.[13] isolated polyaromatic hydrocarbons degrading bacteria from industrial areas (contaminated sites). In the current study, *B. cereus* (KS37) was unable to grow on xylene and toluene, while *B. cereus* (KS1) was unable to grow

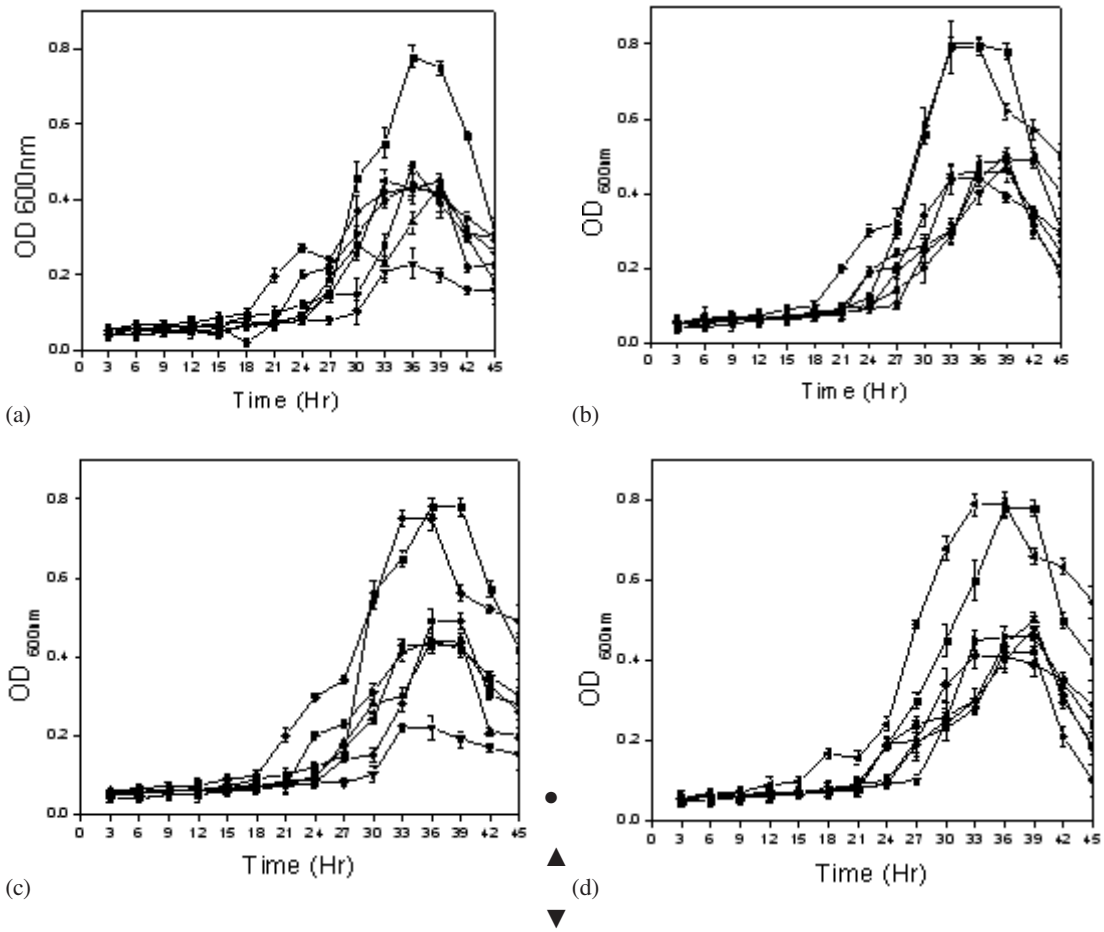

Fig. 1. Growth profile of isolate (a) KS1, (b) KS37, c) KS141 and (d) KS146 in medium supplemented with different concentrations of anthracene or naphthalene. (■) control, ( ) .5mg/50ml Ant, (▲ %) . 8mg/50ml Ant, (▼) 1mg/50ml Ant, (◄►) .5mg/50ml Nap, (◄) .8mg/50ml Nap, (►) 1mg/50ml Nap

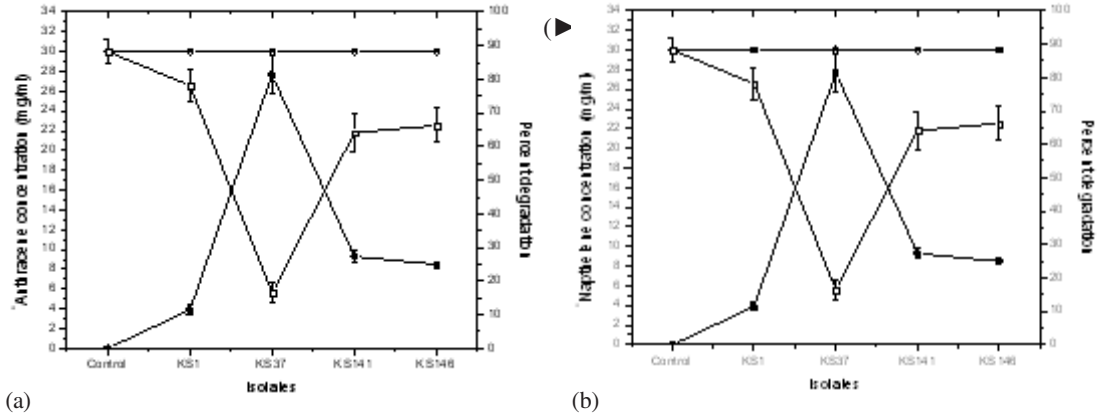

Fig. 2. Degradation of (a) anthracene and (b) naphthalene by rhizospheric isolates from *Calotropis* sp. estimated by HPLC analysis. (ω,) Anthracene and naphthelene at 0 day (Δ) Anthracene and naphthelene at 6 day, (ˆ) % degradation. Error bars indicate standard error of the mean.

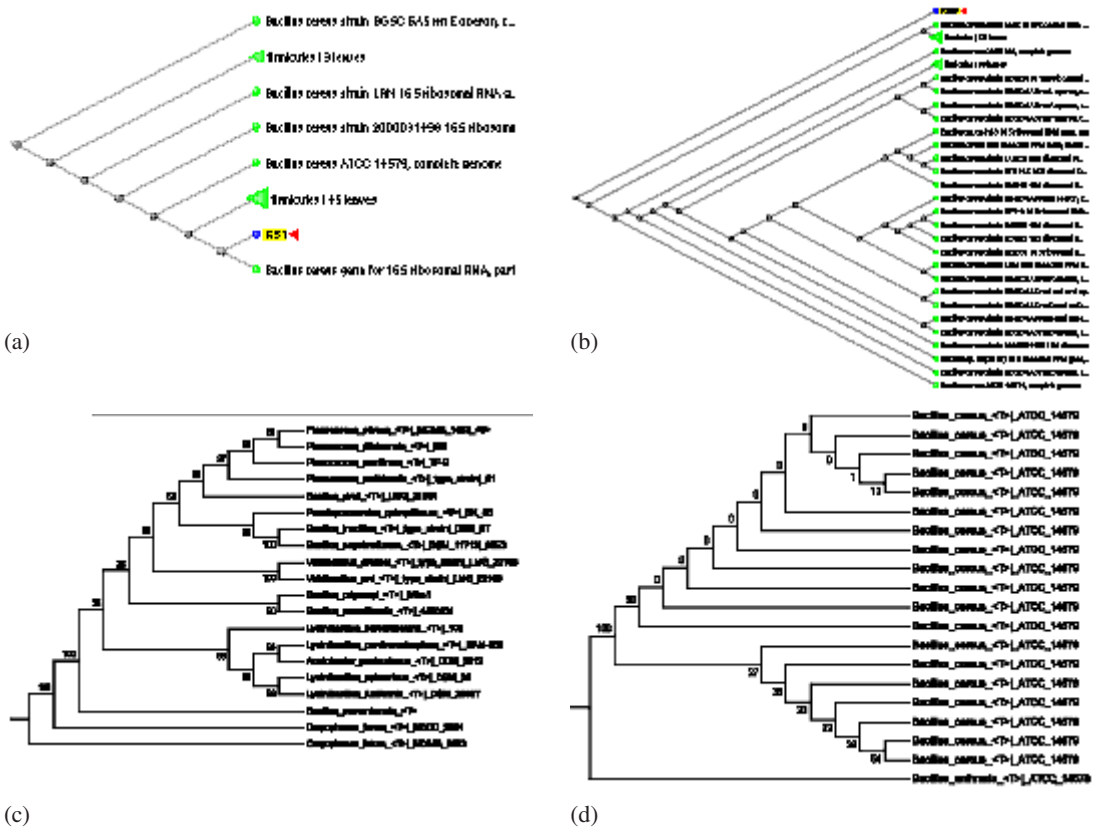

Fig. 3. Neighbour joining phylogenetic trees derived from 16S rRNA gene sequences showing the relationship between isolated strain and related *Bacillus* sp., (a) KS1, (b) KS37, (c) KS141 and (d) KS146

on benzene supplemented medium. A possible reason is the absence of necessary intracellular dioxygenases enzyme for utilization of these hydrocarbons[14].

Efficient degradation of PAHs *in situ* depends not only on the catabolic ability of the strains concerned, but also on the ecological and physiological properties (hydrphobicity and motility) that allow them to survive *in situ*[15]. Therefore, for the strains examined in this study, a direct correlation was found between cell surface hydrophobicity and percentage degradation of PAH. KS37 was able to degrade 81.39% anthracene and also showed 25% hydrophobicity. It has been suggested quite strongly that bacterial strains to be used for bioremediation purposes (especially for bioaugmentation) need to be selected not only on the basis of their catabolic properties *in vitro*, but also with respect to their ecology, to optimize their survival in the target environment[15, 16]. During the course of growth experiment, it was found that the maximum mean growth rate constant of selected isolates was recorded when glucose was used as sole source of carbon while it was found less when anthracene or naphthalene was provided as carbon source. These findings are supported by an earlier study in which successive increase resulted in increased growth rate of isolates with substrate concentration[17]. Juhasz et al.[18] also have studied the effect of PAH on growth of *Burkholderia cepacia*, where growth was evaluated by visual monitoring; whereas in the current study mean growth rate (K) was determined at different concentration of anthracene and naphthalene.

In the present study, all the four isolates (*Bacillus* sp.) were found to substantially reduce PAH concentration in medium as estimated by

HPLC analysis. The results of the present study confirmed the fact that *Bacillus* strains were found to degrade PAHs[19]. Similarly Rochelle et al.[20] showed that the *Bacillus subtilis* is a potential degrader of pyrene and benzo pyrene. The results of present investigation are in contrast with the findings of Hassan et al.[13] and Cerniglia[21], in which PAHs degrading bacterial strains were gram negative and belong to the genus *Pseudomonas*.

All four selected isolates were tentatively classified as *Bacillus* sp. on the basis of morphological and biochemical characterization. A lot of studies on polluted soils undergoing bioremediation have been performed using *Pseudomonas* sp., but very limited literature was reported on the roles of *Bacillus* sp. in hydrocarbon bioremediation. However, there are several reports of bioremediation of polycyclic aromatic hydrocarbon by the action of *Bacillus* sp. occurring in extreme environments[22, 23, 24]. In addition, Ijah and Antai[25] reported *Bacillus* sp. being the predominant isolates of all the crude oil utilizing bacteria characterized from highly polluted soil samples (30 % and 40 % crude oil). *Bacillus* sp. is more tolerant to high levels of hydrocarbons due to their resistant endospores[26]. The isolates, although showed high degree of 16S rRNA sequence similarity with members of *Bacillus cereus* group, in the absence of overall genome comparison data and detailed chemotaxonomic data, they could not be given a species status. These isolates were concluded as *Bacillus* sp. In addition it is worth to note that isolates belonging to *Bacillus* sp. are considered safe for use in biotechnological applications. The results obtained indicate that the rhizosphere of *Calotropis* is a rich source of *Bacillus* sp., which have potential to degrade the PAH molecules.

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